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Disruption of Steroid and Prolactin Receptor Patterning in the Mammary Gland Correlates with a Block in Lobuloalveolar Development

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Targeted deletion of the bZIP transcription factor, CCAAT/enhancer binding protein-β (C/EBPβ), was shown previously to result in aberrant ductal morphogenesis and decreased lobuloalveolar development, accompanied by an altered pattern of progesterone receptor (PR) expression. Here, similar changes in the level and pattern of prolactin receptor (PrlR) expression were observed while screening for differentially expressed genes in C/EBPβ^{null} mice. PR patterning was also altered in PrlR^{null} mice, as well as in mammary tissue transplants from both PrlR^{null} and signal transducer and activator of transcription (Stat) 5a/b-deficient mice, with concomitant defects in hormone-induced proliferation. Down-regulation of PR and activation of Stat5 phosphorylation were seen after estrogen and progesterone treatment in both C/EBPβ^{null} and wild-type mice, indicating that these signaling pathways were functional, despite the failure of steroid hormones to induce proliferation. IGF binding protein-5, IGF-II, and insulin receptor substrate-1 all displayed altered patterns and levels of expression in C/EBPβ^{null} mice, suggestive of a change in the IGF signaling axis. In addition, small proline-rich protein (SPRR2A), a marker of epidermal differentiation, and keratin 6 were misexpressed in the mammary epithelium of C/EBPβ^{null} mice. Together, these data suggest that C/EBPβ is a master regulator of mammary epithelial cell fate and that the correct spatial pattern of PR and PrlR expression is a critical determinant of hormone-regulated cell proliferation. (*Molecular Endocrinology* 16: 2675–2691, 2002)

MOUSE MAMMARY GLAND development occurs postnatally under the control of systemic hormones and local growth factors. Mice are born with a rudimentary ductal structure, and between 3 and 8 wk of age levels of systemic ovarian hormones increase during puberty resulting in the penetration of the ducts into the surrounding fat pad (1). During pregnancy, exposure to estrogen (E), progesterone (P), and the lactogenic hormones, prolactin (Prl) and placental lactogen, induces lobuloalveolar development. By the end of pregnancy, the fat pad is completely filled with secretory epithelium.

Previous studies have determined that the CCAAT/enhancer binding protein-β (C/EBPβ) transcription factor is required for normal ductal morphogenesis and lobuloalveolar development during pregnancy (2, 3). The mammary glands of C/EBPβ^{null} mice exhibit enlarged, cystic ducts with decreased side-branching and an inhibition of alveologenesis in response to E + P. In addition, increased levels of progesterone receptor (PR) mRNA and protein were detected in the mammary epithelial cells (MECs) of C/EBPβ^{null} mice, with an altered distribution of PR-expressing cells along the ducts. This alteration in PR expression correlated with a 10-fold decrease in proliferation induced by an acute, 2-d treatment with E + P (4). This was unexpected because progesterone acts a mitogen to stimulate proliferation of MECs (5), and PR in MECs has been demonstrated to be essential for lobuloalveolar development (6). Furthermore, transplantation experi-

Abbreviations: BrdU, Bromo-deoxyuridine; C/EBPβ, CCAAT/enhancer binding protein-β; DAPI, 4′,6 diamidino-2-phenylindole; E, estrogen; ER, E receptor; IGFBP, IGF binding protein; IRS, insulin receptor substrate-1; ISH, *in situ* hybridization; Jak, Janus kinase; K6, K10, K14, or K18, keratin 6, 10, 14, or 18; MECs, mammary epithelial cells; NKCC1, sodium potassium chloride; P, progesterone; PR, progesterone receptor; Prl, prolactin; PrlR, Prl receptor; RPA, ribonuclease protection assay; SSH, suppression subtractive hybridization; SPRR2A, small proline-rich protein; Stat5, signal transducer and activator of transcription 5; TBS, Tris-buffered saline.

ments with chimeras containing PR^{null} MECs tagged with lacZ along with wild-type MECs indicated that alveolar development in PR^{null} cells occurred when the two cell types were in close proximity, thus demonstrating that PR acts in a paracrine fashion to stimulate proliferation of neighboring cells (7).

The study of gene-targeted mouse models has helped identify other systemic hormone and local growth factors and their cognate receptors required for mammary gland development (reviewed in Ref. 8). Deletion of many of these genes results in mammary gland phenotypes that exhibit defective lobuloalveolar development. For example, the Prl receptor (PrlR) is required for pregnancy-induced lobuloalveolar development. $PrlR^{null}$ mice exhibit a phenotype similar to that observed in the PR^{null} mice (9). This is supported by evidence that PR can regulate PrlR expression (10, 11). The side-branching defect observed in $PrlR^{null}$ mammary glands can be rescued with P treatment, but lobuloalveolar development still did not occur (12).

Signal transducer and activator of transcription 5 (Stat5) is an essential component of the PrlR signal transduction pathway (13). Stat5a-deficient mice exhibit impaired lobuloalveolar development, which could be partially compensated by Stat5b after multiple pregnancies (14, 15). Stat5b, however, was not essential for lactation, and no significant mammary gland phenotype was observed in Stat5b-deficient mice (16). Because of this potential redundancy, a deletion of both Stat5 genes was generated (16). The mammary gland phenotype of these mice was more severe than in Stat5a-deficient mice, with a complete block of lobuloalveolar development and the absence of functional differentiation (17).

Although PR is required for lobuloalveolar development, E receptor α (ERα) is required at an earlier stage to induce ductal elongation (18). Mammary glands from $ER\alpha^{null}$ mice have a rudimentary ductal tree that does not fill the fat pad, although it can undergo lobuloalveolar development in response to a pituitary isograft or E + P treatment (19). In separate studies of the normal mammary gland, it has been shown that PR and ERα are coexpressed in MECs approximately 96% of the time (20). These steroid receptor-positive cells are often located adjacent to proliferating cells but rarely colocalize (4, 20, 21). It is thought that proliferating MECs eventually give rise to more differentiated, steroid receptor-expressing MECs (20, 22).

In the current study, additional changes in gene expression were identified in the mammary glands of $C/EBP\beta^{null}$ mice that provide further insight into the mechanisms by which systemic hormones and local growth factors regulate lobuloalveolar development. Alterations in the level and pattern of PrlR concomitant with the changes in PR were observed in $C/EBP\beta^{null}$ mice. These studies suggest that the correct distribution of both PR and PrlR is required to facilitate hormone-induced proliferation, and that defects in PR expression and proliferation are common features among several mouse models in which impaired lobuloalveolar development is observed. Changes in IGF binding protein (IGFBP)-5, IGF-II, and insulin receptor substrate-1 (IRS-1) expression and alterations in their distribution also were observed in $C/EBP\beta^{null}$ mice, suggesting a role of the IGF axis in the paracrine regulation of lobuloalveolar development. Finally, the inappropriate expression of an epidermal differentiation marker, small proline-rich protein (SPRR2A), and K6 suggests that the germline deletion of C/EBPβ not only disrupts the necessary hormone receptor patterning but also results in an alteration of MEC cell fate.

RESULTS

Coordinate Regulation of PrlR and PR Expression in $C/EBP\beta^{null}$ Mice

To identify additional molecular changes in the mammary glands of $C/EBP\beta^{null}$ mice that might indicate mechanisms by which systemic hormones and local growth factors regulate lobuloalveolar development, a candidate gene approach in which genes known to be important for lobuloalveolar development were examined. PrlR is a likely candidate gene because its expression has been shown to be regulated by progesterone (10, 11), and deletion of PrlR also results in a lack of lobuloalveolar development (9, 23).

Additional evidence that PrlR expression is mediated through activation of PR is shown in Fig. 1A. Semiquantitative RT-PCR analysis of the long form of PrlR was performed using mammary gland RNA from either wild-type or PR^{null} mice at 5 or 12 wk of age. In the mammary glands of wild-type mice there was an approximate 3-fold increase in the amount of PrlR mRNA between 5 and 12 wk of age, coinciding with the increase in circulating ovarian hormones that occurs during this time (1). PR^{null} mice expressed a much lower level of PrlR at 5 and 12 wk of age, suggesting that PrlR expression depends, at least in part, on PR. These data and previous *in situ* hybridization (ISH) studies suggest that PR and PrlR may be coregulated during early mammary gland development (24).

Accordingly, the levels of PrlR mRNA were examined in the mammary glands of $C/EBP\beta^{null}$ and wild-type mice. Northern blot analysis was performed using poly(A) RNA isolated from mice treated acutely with E + P for 2 d to measure the level of PrlR mRNA (Fig. 1B). E, acting through ERα, is known to elevate serum Prl levels (25), and P, acting through PR, increases PrlR mRNA expression (10, 11). A marked increase in the amount of the long form of PrlR mRNA was detected in the mammary glands from $C/EBP\beta^{null}$ mice relative to wild-type mice. Hybridization to a keratin 18 (K18) probe demonstrated both similar mRNA loading and levels of mammary epithelium, which was expected after only 2 d of E + P treatment. ISH was also performed to determine the cellular distribution of PrlR mRNA in $C/EBP\beta^{null}$ mammary glands (Fig. 1, C and D). Signal was detected in a nonuniform, punctate

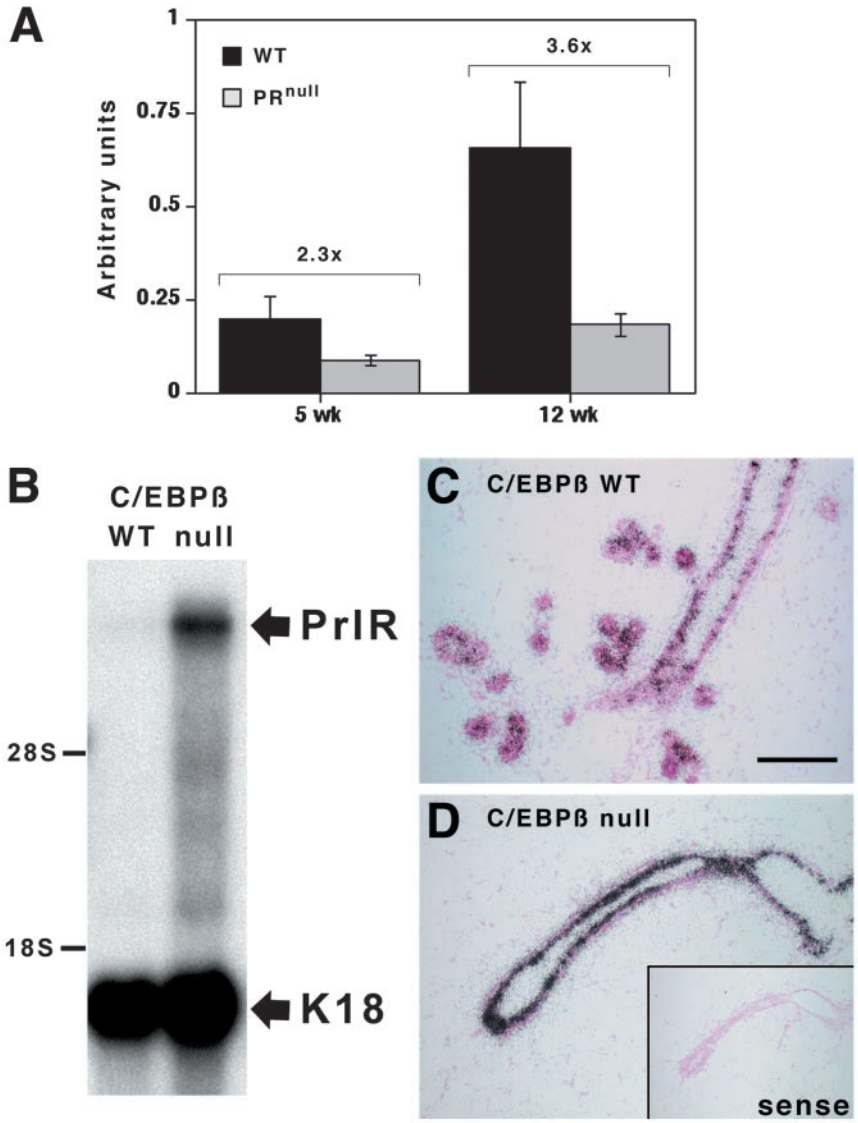

Fig. 1. Altered Expression of PrlR mRNA in Mammary Glands from PR^{null} and C/EBPβ^{null} Mice

Semiquantitative RT-PCR was performed to assess the levels of PrlR in wild-type and PR^{null} mice (A). Mammary tissue from four to six mice for each age and genotype was pooled to isolate total RNA, which was then reverse transcribed and PCR amplified using primers for the long form of PrlR. PrlR levels were normalized to the amount of GAPDH mRNA expressed. Values are representative of three reverse transcription reactions with the *error bars* indicating the SEM. There was a significant difference between the wild-type and PR^{null} samples at 12 wk ($P < 0.05$). Northern blot analysis (B) using 2 μg of poly(A) RNA per lane demonstrated an increase in the long form of PrlR mRNA in the C/EBPβ^{null} mice after treatment with E + P for 2 d. Hybridization to a K18 cDNA probe was used as a control for loading and epithelial cell content. The positions of 18S and 28S rRNAs are indicated. Differences in the levels and cellular distribution of PrlR mRNA were shown by ISH using paraffin-embedded sections from mammary glands treated 2 d with E + P. There was a nonuniform, punctate pattern of expression in wild-type sections (C), but the levels increased and became more uniform in C/EBPβ^{null} mice (D). The *inset* in panel D shows the nonspecific background observed with the control sense riboprobe (*bar*, 200 μm).

pattern in MECs along the ducts in mammary glands from wild-type mice, similar to the pattern observed previously for PR (4, 24). However, PrlR mRNA levels were not only increased in C/EBPβ^{null} mice, but the pattern of expression along the ducts was also more uniform, similar again to alterations observed previously for PR expression (4). Based on the results of the semiquantitative ISH, there was an increased level of PrlR mRNA per cell in the C/EBPβ^{null} gland, in addition to a higher percentage of cells expressing PrlR.

Increased PR and Decreased Proliferation in the Mammary Glands of PrlR^{null} Mice

Because the levels and pattern of PrlR expression were altered in C/EBPβ^{null} mice, and because PR and

PrlR appear to be coordinately regulated, the levels of PR were examined in the mammary glands from intact, untreated PrlR^{null} animals. Between 6 and 9 wk of age, PR expression went from a uniform pattern to a heterogenous, punctate pattern in the MECs of wild-type mice (Fig. 2, A and C), consistent with previous observations (4). By 12 wk, the percentage of PR-positive ductal cells had decreased to approximately 25% (Fig. 2, E and G). In contrast, PR expression was not down-regulated between 6 and 12 wk in PrlR^{null} mice, retaining a level of approximately 50% positive MECs (Fig. 2, B, D, and F). The difference in the percent of

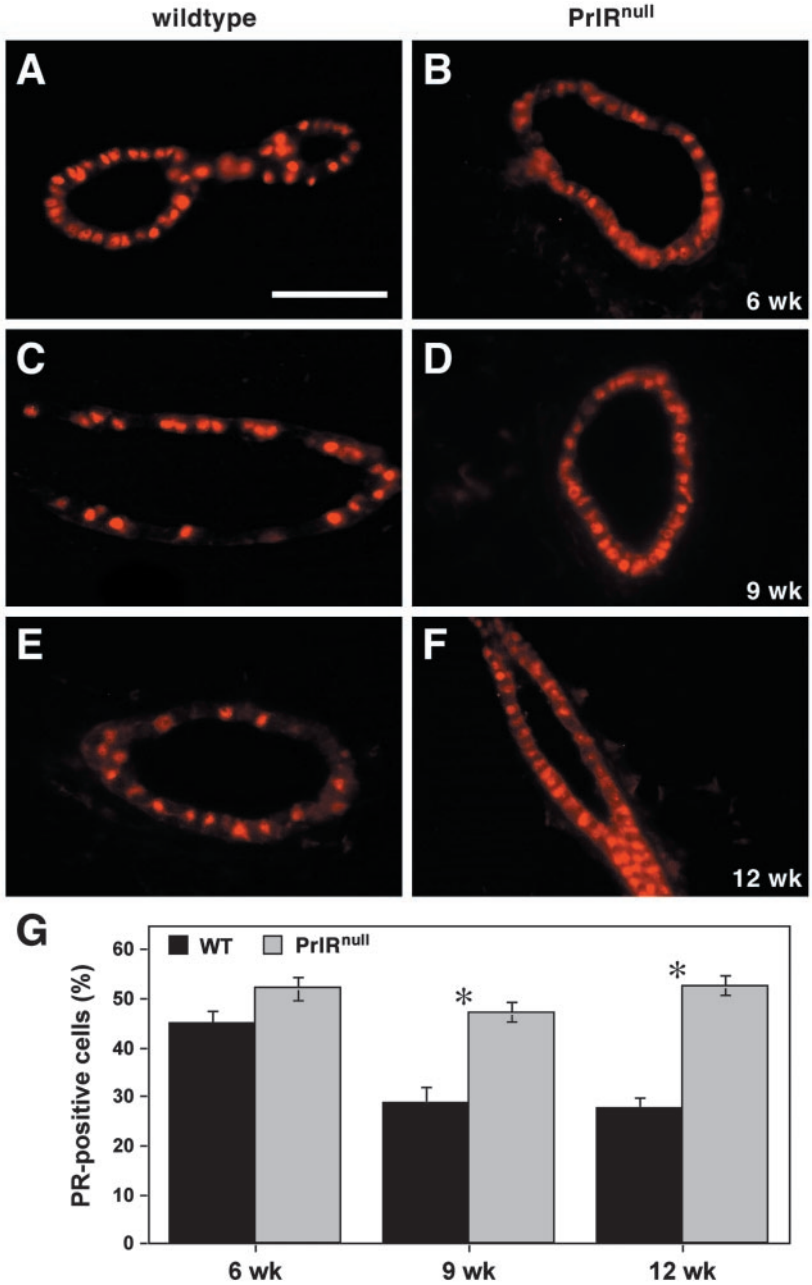

Fig. 2. Disrupted Pattern of PR Expression during Development of Mammary Glands in Nulliparous PrlR^{null} Mice

Immunofluorescence staining for PR was performed on sections of untreated wild-type or PrlR^{null} glands taken at 6 (A and B), 9 (C and D), and 12 (E and F) wk of age. PR expression was down-regulated in the wild-type between 6 and 9 wk, and appeared nonuniform by 12 wk (A, C, E). PR expression remained elevated in the PrlR^{null} MECs during this time frame (B, D, F). The images were taken at ×60 magnification (*bar*, 50 μm). Quantitation of PR-positive MECs is plotted in the *bar graph* (G), with the *error bars* showing the SEM. Statistically significant differences were observed between wild-type and PrlR^{null} mice at 9 and 12 wk of age (indicated by the *asterisks*). Four animals from each age group and genotype were analyzed, and an average of 1400 nuclei were counted for each data set.

PR-positive MECs between the two genotypes was statistically significant at 9 and 12 wk of age (Fig. 2G).

Because an increase in PR expression was associated with a decrease in the number of proliferating MECs in mature C/EBPβ^{null} animals after an acute E + P treatment (4), the level of proliferation was also examined in PrlR^{null} mice. PR expression and bromodeoxyuridine (BrdU) incorporation were analyzed in mammary glands from 12-wk PrlR^{null} animals that were treated for 2 d with E + P. As above, there was an increased percentage of PR-positive MECs in PrlR^{null} mice (Fig. 3B) as compared with wild-type animals (Fig. 3, A and C). When the incorporation of BrdU was measured, there was a significant decrease in the number of proliferating MECs in the PrlR^{null} mice (Fig. 3, E and F). The finding that 13% of MECs were BrdU-positive in wild-type mice (Fig. 3, D and F) was consistent with previous results (4).

Transplanted PrlR^{null} and Stat5ab-Deficient Mammary Tissue Also Exhibit Increased PR and Decreased Proliferation

Stat5 is a signaling molecule downstream of PrlR, and is important for lobuloalveolar development. Because Stat5ab-deficient and PrlR^{null} mice are infertile and

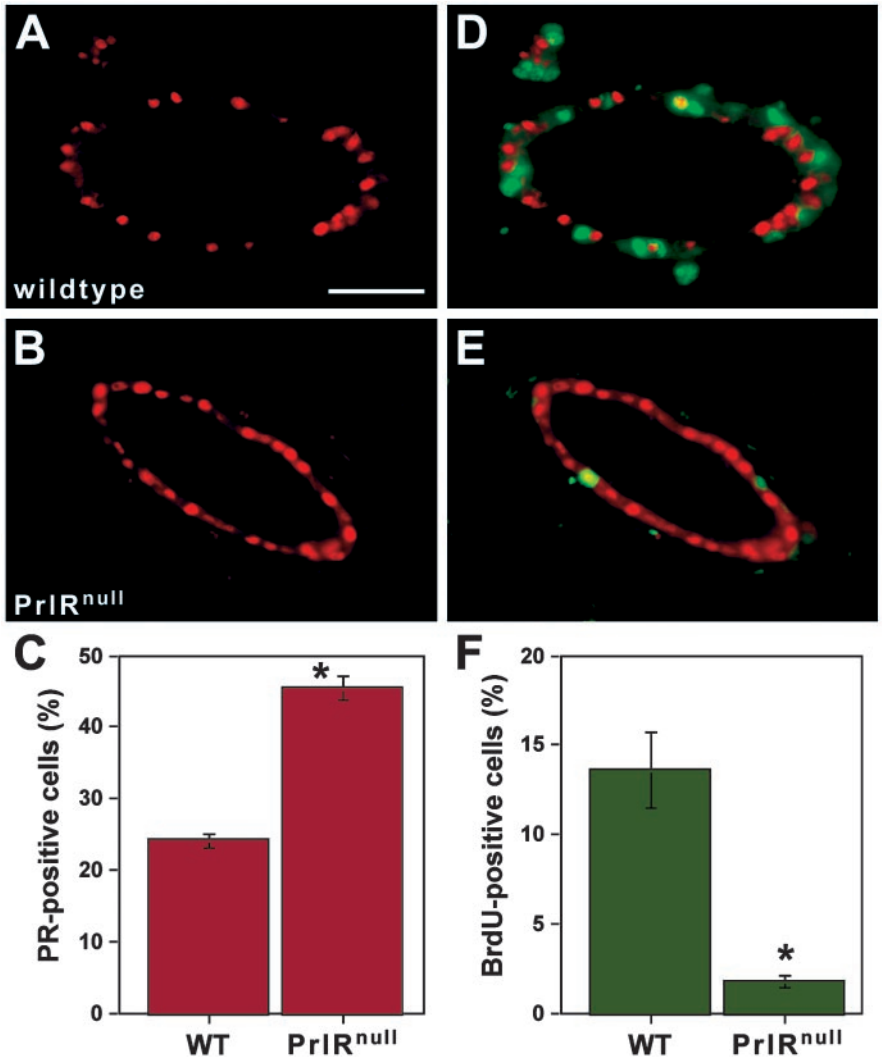

Fig. 3. Increased PR Expression and Decreased Proliferation in the Ductal Epithelium of Mammary Glands from PrlR^{null} Mice after 2 d of E + P

Immunofluorescence staining for PR (*red*) and BrdU (*green*) was performed on mammary gland sections from 12-wk-old wild-type or PrlR^{null} mice after treatment for 2 d with E + P. PR expression was increased and proliferation was decreased in the PrlR^{null} (B, E) compared with wild-type (A, D). Note the PR- and BrdU-positive cells rarely colocalized. The images were taken at ×60 magnification (*bar*, 50 μm). Quantitation of PR- and BrdU-positive MECs is plotted in the *bar graphs* (C and F), with the *error bars* showing the SEM. Statistically significant differences were observed between wild-type and PrlR^{null} for both PR and BrdU ($P < 0.0001$; indicated by the *asterisks*). Four animals from each group were used and an average of 1400 nuclei were counted for each data set.

have deficiencies in circulating ovarian hormones (9, 16), mammary tissue from both genotypes was transplanted into the cleared fat pads of recipient nude mice. This approach permitted a direct determination of the effects of these gene deletions on MECs in the absence of any systemic effects, as well as a direct comparison of the $PrlR^{null}$ and Stat5ab-deficient MECs exposed to the same hormonal milieu in the same host animal. Nine to 10 wk after transplantation, host animals were treated for 2 d with E + P and the outgrowths collected. Analysis of PR expression revealed that the percent of PR-positive MECs in the endogenous no. 3 control gland was significantly higher than the value of 25% usually observed (Fig. 4A). When mammary gland tissue from wild-type mice was transplanted into nude mice and analyzed for PR expression, the percent PR-positive MECs closely resembled the endogenous gland shown in Fig. 4A (data not shown), suggesting that the lowered levels of circulating E + P in nude mice (26) accounted for the higher level of PR in the controls. Despite the higher basal level of PR expression in nude mice, both Stat5ab-deficient and $PrlR^{null}$ epithelium had increased levels of PR-positive MECs relative to the control (Fig. 4, B and C). There were statistically significant differences between the two gene-deleted outgrowths and the control, as well as between Stat5ab-deficient and $PrlR^{null}$ outgrowths (Fig. 4D). Proliferation in Stat5ab-deficient outgrowths was also lower relative to the control (Fig. 4, F *vs.* E), but not to the extent in $PrlR^{null}$ outgrowths (Fig. 4G). This difference is most likely because PrlR acts through multiple signaling pathways, including Janus kinse (Jak) 2, phosphatidylinositol-3 kinase, and MAPK, as discussed previously (17). In all cases, the amount of PR expressed was inversely proportional to the rate of proliferation.

Altered Expression and Localization of IGF Axis Molecules in Mammary Glands of C/EBPβ^{null} Mice

Suppression subtractive hybridization (SSH) PCR was performed to identify differentially regulated genes from the mammary glands of mature, nulliparous C/EBPβ^{null} mice (3–6 months old) as compared with wild-type mice (27). At this stage of development, differences in gene expression should reflect changes in ductal morphology in these mice, rather than alterations in the epithelial/stromal cell ratio. Several hundred clones were randomly chosen for high throughput reverse Southern blotting (WT-subtracted, 200 clones; C/EBPβ^{null}-subtracted, 350 clones). To identify potentially differentially expressed genes, these clones were probed with enriched total cDNAs prepared from mammary glands of either wild-type or C/EBPβ^{null} mice. The 60 clones identified by this method were then sequenced. A summary list of selected genes identified in this screen is published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org.

One of the genes identified was IGFBP-5. IGFBP-5 was also identified during a screen using the CLONTECH Laboratories, Inc. (Palo Alto, CA) Atlas array as a gene down-regulated in the mammary glands of C/EBPβ^{null} mice (data not shown), and this observation was confirmed by Northern blot analysis of mammary gland mRNA from untreated C/EBPβ^{null} mice (Fig. 5A). ISH was performed on frozen sections of mammary glands from untreated, nulliparous wild-type or C/EBPβ^{null} mice to determine the cellular distribution of the IGFBP-5 mRNA (Fig. 5, B–E). Opposite to the pattern of PR and PrlR expression, IGFBP-5 mRNA changed from a relatively uniform pattern of expression in wild-type mammary glands to a nonuniform, punctate pattern in C/EBPβ^{null} mice. This effect was more pronounced in the C/EBPβ^{null} after treatment for 2 d with E + P (Fig. 5, C and E).

Because of the alteration in IGFBP-5 expression, we examined the expression of two other molecules in the IGF signaling axis: IGF-II and IRS-1. Frozen sections of mammary glands from untreated 6-wk or 12-wk animals or 2-d E + P-treated mature mice were analyzed for IGF-II expression by ISH (Fig. 5, F–K). At 6 wk, expression of IGF-II was uniform in the ductal epithelium from both wild-type and C/EBPβ^{null} mice (Fig. 5, F and I). By 12 wk, the pattern of expression became punctate in the wild-type (Fig. 5G) but remained uniform in the ducts of C/EBPβ^{null} mice (Fig. 5J), reminiscent of the change in patterning of PR and PrlR expression. The difference in IGF-II distribution became more pronounced in wild-type mice after 2 d of E + P treatment (Fig. 5H).

IRS-1 is a cytoplasmic signaling molecule downstream from the insulin and IGF-I receptors. No significant differences in IRS-1 mRNA levels were observed between untreated or 2-d E + P-treated wild-type and C/EBPβ^{null} mice (Fig. 5L) as determined by ribonuclease protection assay (RPA). However, when the expression of IRS-1 protein was analyzed by Western blot, a 2- to 3-fold decrease between wild-type and C/EBPβ^{null} mice was observed in both the untreated and 2-d E + P-treated animals (Fig. 5M). A change in the expression pattern of IRS-1 from uniform in the wild-type (Fig. 5N) to nonuniform in the ducts of C/EBPβ^{null} mice (Fig. 5O) was observed by immunostaining and correlated with the results obtained by Western blotting.

Hormonally Regulated Signaling Pathways in the Mammary Glands of C/EBPβ^{null} Mice Are Functional

Whereas the levels of PR and PrlR increased in the mammary glands of C/EBPβ^{null} mice, it is not clear if the signaling pathways in these cells are functional. Turnover and down-regulation of PR protein results from prolonged exposure to P (28, 29). Therefore, wild-type and C/EBPβ^{null} mice were implanted with

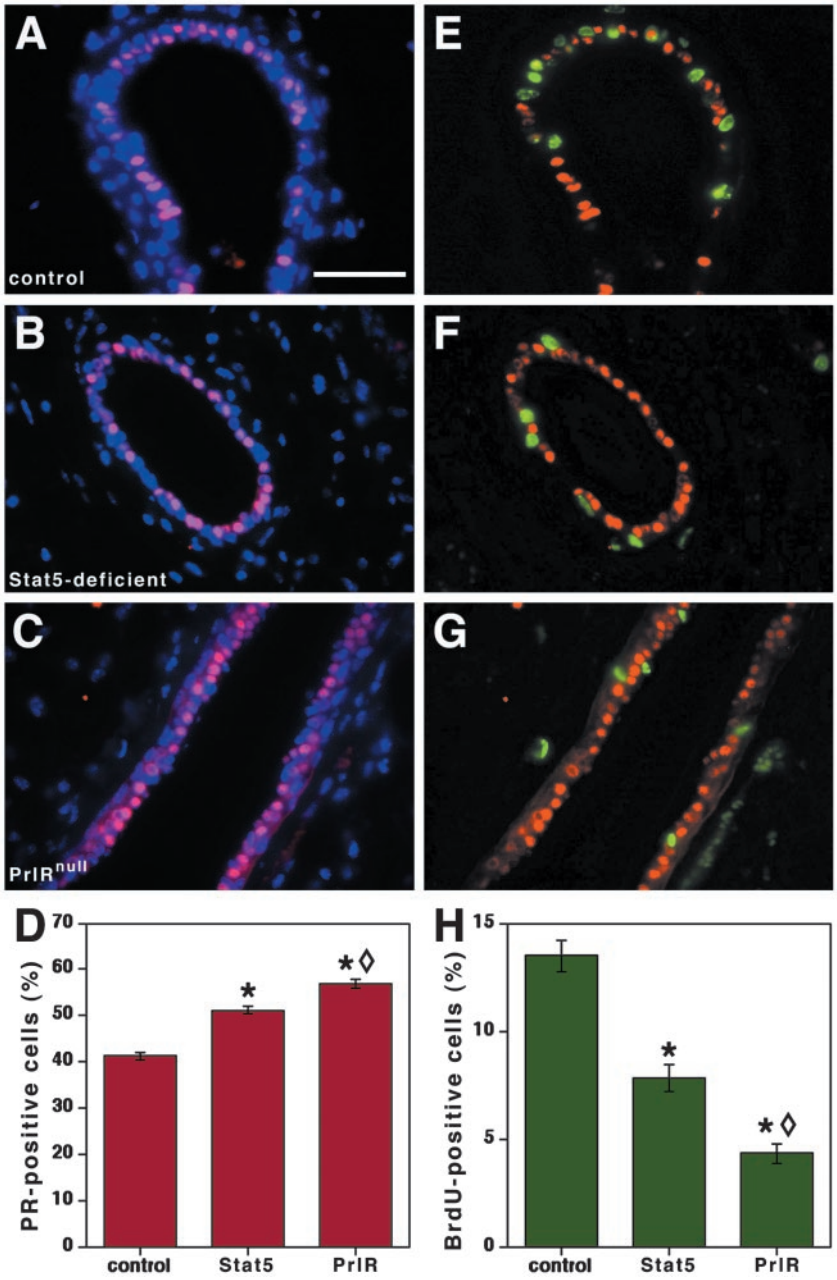

Fig. 4. Increased PR and Decreased Proliferation in Mammary Outgrowths from Stat5ab-Deficient and PrlR^{null} Mammary Tissue Transplants

Nine to 10 wk after transplantation, host mice were treated for 2 d with E + P. Stat5ab-deficient and PrlR^{null} transplants and the endogenous no. 3 mammary glands (control) were analyzed for PR staining (*red*) and BrdU incorporation (*green*). The nuclei were stained with DAPI (*blue*). PR expression was increased and proliferation was decreased in both the Stat5ab-deficient (B and F) and PrlR^{null} (C and G) outgrowths, as compared with the control (A and E). The images were captured at ×60 magnification (*bar*, 50 μm). The percentage of PR- and BrdU-positive MECs is plotted in the *bar graphs* (D and H), with the *error bars* showing the SEM. Statistically significant differences were observed between control and Stat5ab-deficient or PrlR^{null} samples for both PR and BrdU (indicated by the *asterisks*). There were also statistically significant differences between Stat5ab-deficient and PrlR^{null} samples (*diamond symbols*). A Student's *t* test was used to analyze the data ($P < 0.0001$). Between 5 and 7 tissue samples were analyzed, and at least 6000 nuclei were counted for each genotype.

E + P pellets for 21 d to determine if PR levels could be down-regulated after chronic exposure to steroid hormones. Comparing the percentage of PR-positive MECs from untreated animals with those treated for 21 d with E + P showed a 2-fold decrease in the glands from wild-type mice (Fig. 6, A *vs.* B). Although the level of PR was almost three times greater in C/EBPβ^{null} mice before treatment, the fold down-

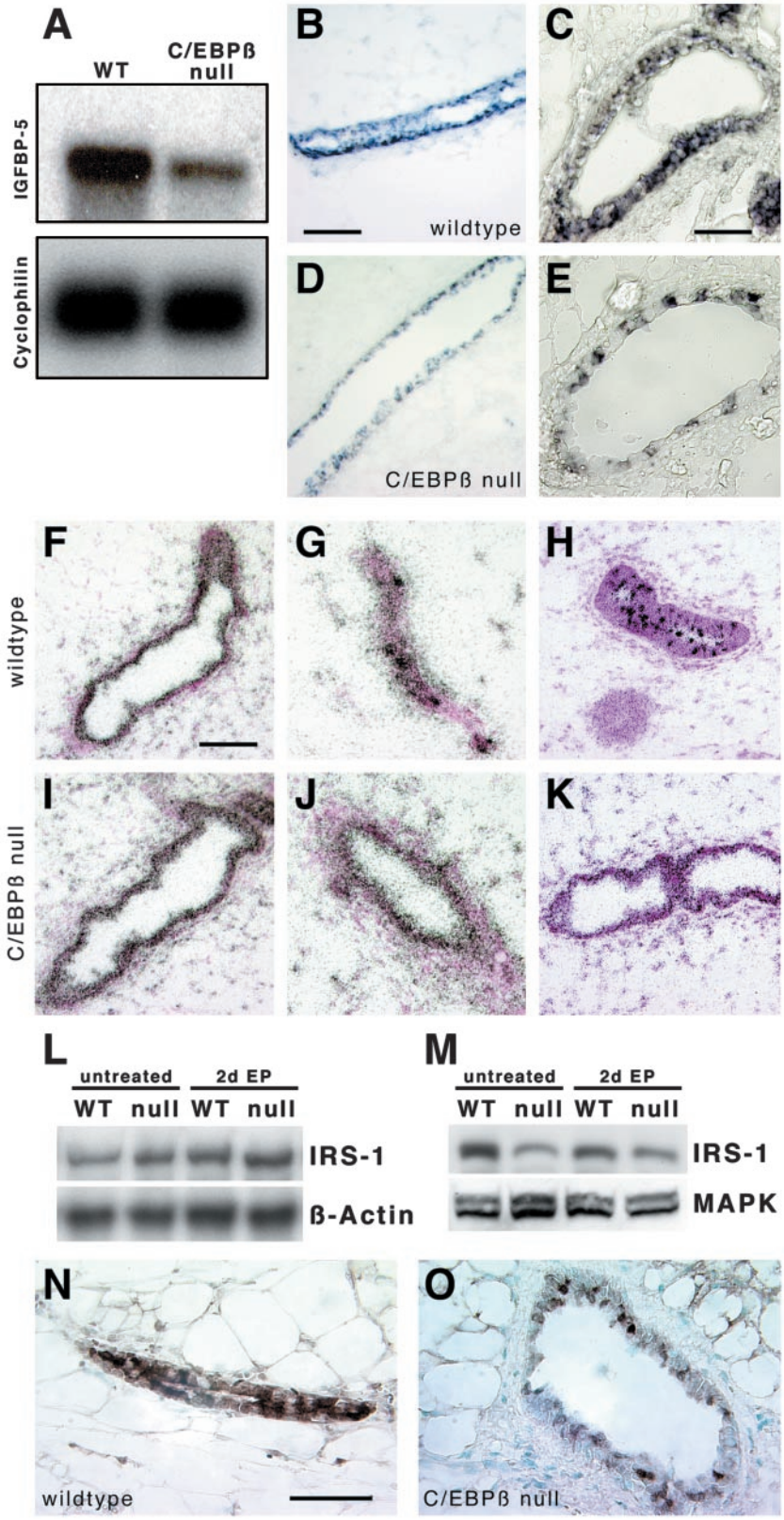

Fig. 5. Disrupted Expression of IGF Axis Molecules in the Mammary Glands of C/EBPβ^{null} Mice

Northern blot analysis (A) demonstrated a decrease in the amount of IGFBP-5 mRNA from the mammary glands of mature, untreated C/EBPβ^{null} mice, as compared with wild type. Cyclophilin mRNA was used as a loading control. Nonradioactive ISH on frozen sections showed that IGFBP-5 mRNA localizes mainly in the epithelial cells. In untreated mice (B and D), the pattern

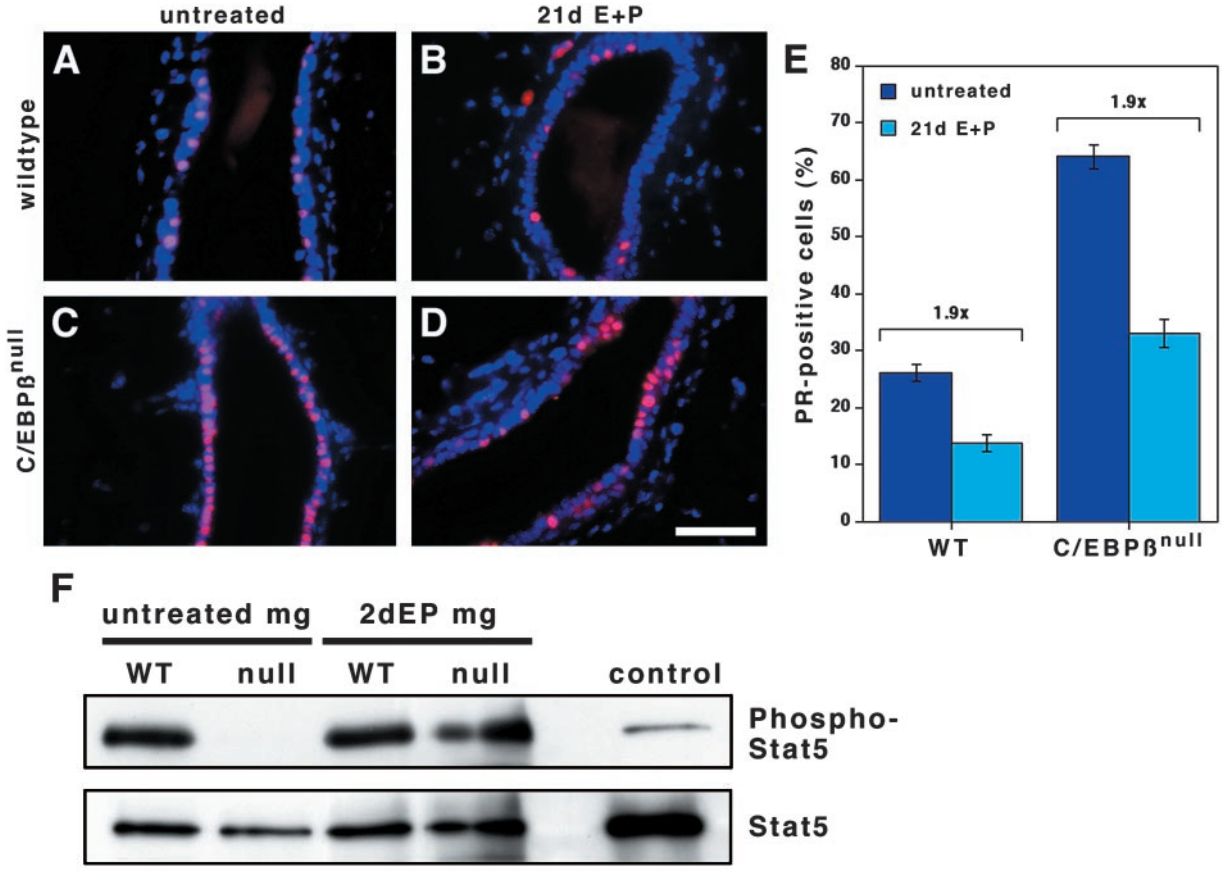

Fig. 6. Hormone-Induced Signaling Pathways Are Intact in C/EBPβ^{null} Mammary Ductal Epithelium

Immunofluorescence staining for PR was performed on sections of mammary gland from wild-type (A and B) or C/EBPβ^{null} (C and D) mice biopsied either after no hormone treatment (A and C) or after treatment for 21 d with an E + P pellet (B and D). DAPI staining of nuclei is shown in *blue* and PR-positive cells are *red*. Images were taken at ×60 magnification (*bar*, 50 μm). Quantitation of PR-positive MECs is plotted in the *bar graph*, with the *error bars* showing the SEM (E). The fold decrease in the percent of PR-positive cells after hormone treatment is similar for both wild type and C/EBPβ^{null}. Four to five animals (ages 18–22 wk) from each genotype and treatment were analyzed, and at least 2700 nuclei were counted for each group. F, Stat5 was immunoprecipitated from 1.5 mg of whole cell extract and blotted with an antiphospho-tyrosine antibody (*top*). The blot was then stripped and reprobed with an anti-Stat5 antibody (*bottom*). The control lane contains whole cell extract from HeLa cells transfected with a Stat5a expression construct and treated with Prl. mg, Mammary gland; null, C/EBPβ^{null}.

regulation after hormone treatment was identical (Fig. 6, C *vs.* D).

Prl acts through the PrlR to activate the Jak/Stat pathway leading to the tyrosine phosphorylation of Stat5. The levels of Stat5 tyrosine phosphorylation were determined by immunoprecipitation-Western blot analysis of whole cell mammary gland extracts from both wild-type and C/EBPβ^{null} mice, either untreated or treated for 2 d with E + P (Fig. 6F). The antibody used recognizes both isoforms, but Stat5a is the predominant form in the mammary gland (15). Levels of Stat5 tyrosine phosphorylation were similar in extracts

of expression changed from uniform in the ductal epithelium of the wild-type gland (B) to nonuniform in ducts from C/EBPβ^{null} mice (D). This pattern was more pronounced after treatment for 2 d with E + P (C and E). Radioactive ISH for IGF-II was performed on frozen sections from untreated animals at 6 wk (F and I) and 12 wk (G and J) or mature animals treated for 2 d with E + P (H and K). The expression of IGF-II at 6 wk was comparable between wild type (F) and C/EBPβ^{null} (I). At 12 wk, IGF-II mRNA levels decreased and assumed a punctate distribution in wild-type MECs (G), but remained uniformly expressed in C/EBPβ^{null} mice (J). The punctate pattern was more pronounced in the ducts from wild-type mice after 2 d E + P treatment (H). The images (B–K) were taken at ×40 magnification (*bar*, 50 μm). A detectable difference in IRS-1 protein levels between wild type and C/EBPβ^{null} was observed by Western blot analysis, with MAPK used as a loading control (M). However, there was no change in IRS-1 mRNA levels, as determined by RPA using β-actin mRNA as a loading control (L). IRS-1 immunohistochemistry demonstrated a uniform expression pattern in the ducts from wild-type mice (N), but expression was decreased and nonuniform in the ducts of C/EBPβ^{null} mice (O). Images were taken at ×60 magnification (*bar*, 50 μm).

from glands from either untreated or hormone-treated wild-type mice. Interestingly, no Stat5 tyrosine phosphorylation was detected in mammary gland extracts from untreated C/EBPβ^{null} mice, but phosphorylation was observed after acute hormone treatment, which may be due to estrogen-induced Prl expression (25).

SPRR2A, a Marker of Epidermal Differentiation, and K6 Are Expressed in the Mammary Glands of C/EBPβ^{null} Mice

Surprisingly, another gene identified by the SSH-PCR screen was SPRR2A, a marker of epidermal differentiation that is normally expressed in the cornified layer of the skin (30). Northern blot analysis confirmed the differential expression of SPRR2A and demonstrated that the level of SPRR2A mRNA was markedly increased in the mammary glands from nulliparous C/EBPβ^{null} mice (Fig. 7A). Immunohistochemistry was performed to determine the levels and pattern of SPRR2 protein expression in the mammary gland (Fig. 7B). There was no staining in wild-type glands but a punctate, nonuniform pattern of expression was observed in the MECs from C/EBPβ^{null} glands, similar to the pattern seen for PR and PrlR. Although this staining was performed on tissue treated for 2 d with E + P, similar results were seen in tissues from untreated animals (data not shown).

This finding led us to investigate whether other epidermal markers, such as keratins, were expressed in the mammary glands of C/EBPβ^{null} mice. K10 staining was negative in both wild-type and C/EBPβ^{null} glands, but was positive on a section of newborn skin (data not shown). K6 is involved in wound healing and is associated with hyperproliferative diseases (31). Whereas K6 is expressed in the body cells of the terminal end buds in the pubertal mammary gland, it is rarely detected in ducts of the mature gland (32, 33). Consistent with these findings, we saw no K6 immunoreactivity in wild-type MECs (Fig. 7C). However, K6-positive MECs were readily apparent in ducts from mature C/EBPβ^{null} mice. Whereas the immunoreactivity was more uniform than that observed for SPRR2A, not all MECs were K6-positive. In contrast, no K6-positive cells were identified in ducts from transplanted PrlR^{null} or Stat5ab-deficient tissue (data not shown). K14 expression is normally detected in the myoepithelial cells surrounding the ducts, and no differences were detected in the staining patterns in the myoepithelium between wild-type and null mice (Fig. 7D).

DISCUSSION

Correct PR and PrlR Patterning Is Required for Normal Lobuloalveolar Development

These studies illustrate the importance of establishing the correct pattern of PR and PrlR expression during ductal morphogenesis to facilitate the proliferative response to steroid and lactogenic hormones during pregnancy. Disruption of PR and PrlR patterning and a concomitant decrease in proliferation was observed in mammary glands from several different gene targeted mouse models, all of which display defects in lobuloalveolar development. Although it has not yet been definitively established that PR and PrlR are expressed in the same cells, ISH experiments using serial sections have demonstrated a very similar pattern of expression for both mRNAs (24). In the current experiments, the level of PrlR mRNA was substantially reduced in the PR^{null} mice, particularly at 12 wk of age when PrlR expression is normally increased in response to rising circulating ovarian hormone levels (1). PrlR mRNA is uniformly expressed in the MECs before puberty, and its expression becomes heterogeneous in the mature gland, similar to the pattern reported for PR during the same time period (24). However, in C/EBPβ^{null} mice, PrlR mRNA remains uniformly expressed in mature mammary glands.

More recently, it has been possible to detect the pattern of PR expression in mice using β-galactosidase staining wherein lacZ has been inserted in place of the PR gene (34). The pattern of lacZ staining observed in mature nulliparous mice was also heterogeneous and was regulated by ovarian steroids during normal development. Furthermore, PR expression has even been observed in the mammary anlage as early as embryonic d 14 (34).

When proliferation rates were quantitated after acute E + P treatment, the Stat5ab-deficient and PrlR^{null} outgrowths contained fewer BrdU-positive MECs than wild-type mice. However, even with fewer BrdU-positive MECs, the dissociation between PR expression and proliferation was maintained in these gene-targeted mouse models. It has been proposed that p27^{Kip1} expression in steroid-receptor positive cells may block cell division (35). Our preliminary results show an increase in the number of p27 positive cells along the ducts of the C/EBPβ^{null} glands (Grimm, S. L., and J. M. Rosen, unpublished observation), which correlates both with the increased PR expression and decreased proliferation.

Although the MECs from C/EBPβ^{null}, PrlR^{null} and Stat5ab-deficient mouse models all display a decreased proliferative response to steroid hormones, the inability of these cells to proliferate is probably not due to defective signaling pathways. The down-regulation of PR protein after prolonged exposure to P is mediated through serine phosphorylation by MAPK, resulting in the ubiquitin-mediated degradation of PR (29). Even though C/EBPβ^{null} mice contain approximately three times the number of PR-positive MECs, chronic exposure to E + P resulted in a 2-fold down-regulation of PR in both wild-type and C/EBPβ^{null} mice. These data rule out the possibility that defects in this pathway account for the increased level of PR in the C/EBPβ^{null} mammary gland. The PrlR/Stat5 pathway also appears to be functional in C/EBPβ^{null} mice. There was no detectable tyrosine phosphorylation of

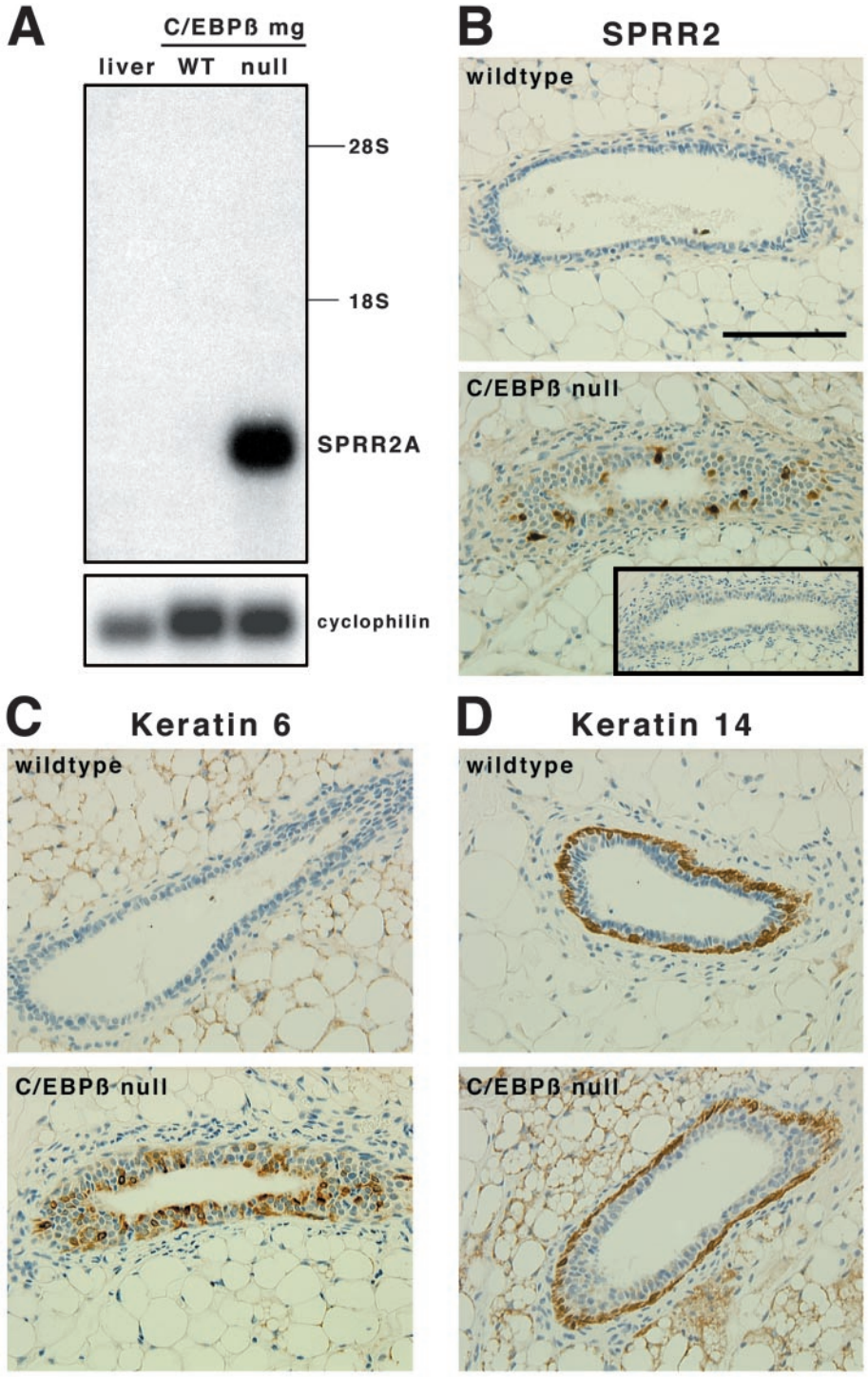

Fig. 7. Expression of SPRR2A, an Epidermal Differentiation Marker, and K6 in the Mammary Glands from C/EBPβ^{null} Mice

Northern blot analysis (A) demonstrated a substantial increase in the amount of SPRR2A mRNA in mammary glands from untreated C/EBPβ^{null} compared with wild-type mice. Cyclophilin mRNA was used as a loading control. Staining for SPRR2 (B) and K6 (C) by immunohistochemistry was observed in C/EBPβ^{null} sections treated for 2 d with E + P, but not in wild-type sections. The *inset* in panel B shows a control where no primary antibody was added. K14 immunostaining (D) of the myoepithelium surrounding the ducts was as expected for both wild-type and null tissues. Images were taken at ×40 magnification (*bar*, 100 μm).

Stat5 observed in the mammary glands of untreated C/EBPβ^{null} mice, most likely due to defective ovarian function in the C/EBPβ^{null} mice (36). However, Stat5 protein was tyrosine phosphorylated after acute hormone treatment, most likely as a consequence of E-induced Prl production (25). However, chronic hor-

mone treatment, which can activate Stat5, was not sufficient to rescue lobuloalveolar development, suggesting that other downstream responses to the Jak/Stat pathway might be altered in C/EBPβ^{null} mice.

These observations have led to the development of a testable autoregulatory model depicted in Fig. 8. During embryonic development, epithelial/mesenchymal interactions required for the development of the mammary anlage may lead to the expression of PR. PR expression then results in the induction of PrlR; whether this occurs via a direct transcriptional mechanism or via an indirect mechanism remains to be determined. PrlR may then act through the Jak/Stat pathway to help regulate the level of ERα expression. This is consistent with the observation that Prl can activate ERα transcription in corpus luteum via Stat5a or 5b (37). Whether Prl directly activates ERα transcription in MECs is not known. Additionally, PrlR may feed back to control PR expression. Finally, ERα may then regulate the level of PR gene transcription both via indirect interactions with other transcription factors, such as SP1, as well as binding to ERα response element half-sites (38, 39). Although this model helps explain the coregulation of ERα, PR, and PrlR observed in MECs in mature, nulliparous mice, the mechanism by which the nonuniform pattern of receptor expression is established in response to E and P remains to be determined. C/EBPβ does not appear to play a direct role in this autoregulatory loop; the level of C/EBPβ mRNA does not change in the PR^{null} mammary gland (4), nor does it differ in transplanted PrlR^{null} or Stat5ab-deficient tissue at parturition (17). Instead, C/EBPβ appears to act at a much earlier stage of development and may be required for the specification of mammary epithelial progenitors.

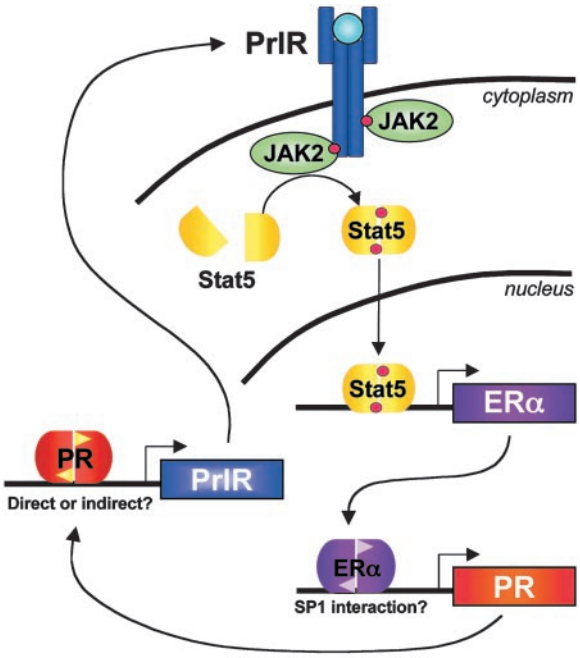

Fig. 8. Testable Model of Autoregulation between PR, PrlR, and ERα Expression

Potential autoregulatory loop between PR, PrlR, and ERα. PR can up-regulate PrlR expression, and there may be feedback from PrlR back to PR. PRL-mediated activation of the Jak/Stat pathway, through phosphorylation of Stat5, may then activate ERα in the mammary gland, which in turn leads to increased PR expression.

Altered Gene Expression in C/EBPβ^{null} Mice

PR and PrlR were not the only genes whose levels and patterns of expression were altered in the ductal epithelium of C/EBPβ^{null} mice. A decreased level of IGFBP-5 mRNA coupled with a shift to a nonuniform, punctate pattern of expression was observed in the mammary ducts of C/EBPβ^{null} mice. These changes were exactly opposite from those observed for PR and PrlR. Little is known concerning the function of IGFBP-5 during early mammary gland development, although this protein is expressed during this period (40). The down-regulation of IGFBP-5 in the mammary glands of C/EBPβ^{null} mice was coincident with the decrease in proliferation observed, suggesting that IGFBP-5 might facilitate the proliferative response. This initially seemed counterintuitive because IGFBP-5 expression in the mammary gland has been mainly associated with increased apoptosis during involution. IGFBP-5 levels increase markedly at the onset of involution in response to the activation of Stat3 (41), and IGFBP-5 is thought to function to sequester IGF-I, which acts as a cell survival factor during lactation (42).

However, IGFBP-5 has been shown previously to augment the effects of IGF-I on the migration and proliferation of smooth muscle cells (43). Recent studies have also demonstrated a positive role for IGFBP-5 in *Xenopus* neural tube induction (44). In this system, it has been suggested that IGFBP-5 potentiates the activity of endogenous IGFs to facilitate signaling through the IGF receptor.

During lactation, Prl is thought to suppress IGFBP-5 expression possibly through the activation of Stat5 (42). The increase in PrlR levels observed in C/EBPβ^{null} mice might, therefore, result in the decreased levels of IGFBP-5 mRNA expression. Changes in the level of IGFBP-5 expression may also reflect the deletion of C/EBPβ, which may act as a direct regulator of IGFBP-5 transcription in the mammary gland, as it does in osteoblasts (45). Again, the mechanisms responsible for the observed changes in the spatial pattern of IGFBP-5 expression remain to be established.

Along with changes in IGFBP-5, the expression patterns of other molecules in the IGF axis were altered in C/EBPβ^{null} mice. IGF-II patterning in wild-type mice was reminiscent of PR and PrlR, with a uniform pattern of expression detected initially at 6 wk that became punctate by 12 wk. However, the pattern of IGF-II mRNA expression remained uniform in C/EBPβ^{null} mice throughout this period of development. IGF-II is known to be a mitogen in the mammary gland

(reviewed in Ref. 40) and may be a direct target of the Prl-induced Jak/Stat pathway (Ormandy, C., unpublished). Whereas the level of IGF-II mRNA was increased at 12 wk post partum in C/EBPβ^{null} mice as compared with wild-type, the decreased expression of IGFBP-5 and IRS-1 may inhibit the paracrine effects of IGF-II in MECs, as well as IGF-I in the mammary stroma.

IRS-1 expression was also decreased in the mammary glands of C/EBPβ^{null} mice. Again, the change in the pattern of IRS-1 expression resembled that observed for IGFBP-5 expression. The decrease in IRS-1 protein expression appears to be regulated at the post-transcriptional level, as reported previously during normal mammary gland development (46). However, this is probably not the result of ligand-mediated activation of IRS-1 (47), based on the marked inhibition of E + P-induced proliferation in the C/EBPβ^{null} mice. With the availability of epitope-specific antibodies directed against the myriad of phosphotyrosines and phosphoserines in IRS-1, it may be possible to determine directly the mechanisms responsible for the different patterns of IRS-1 expression observed in the mammary gland.

Additional evidence for involvement of the IGF axis with PrlR signaling comes from comparing RNA isolated from transplanted mammary tissue from wild-type and PrlR^{null} mice using Affymetrix microarrays. IGF-II expression was decreased at d 6 of pregnancy in the PrlR^{null} transplants, with no change observed at d 2 or 4 of pregnancy (Ormandy, C., unpublished). Thus, it is conceivable that IGF-II may act as one of several local growth factors, including Wnt-4 (48, 49), receptor activator of nuclear factor κB ligand (50), TGFα, and/or amphiregulin (51), all of which may be important mediators of the paracrine/juxtracrine action of steroid hormones and Prl on proliferation during lobuloalveolar development.

Altered Cell Fate in the Mammary Glands of C/EBPβ^{null} Mice

These results suggest that the germline deletion of C/EBPβ may result in a change in cell fate preventing ductal epithelial progenitors from responding appropriately to hormone-regulated signal transduction pathways. In this regard, one of the genes identified in the SSH screen that is up-regulated in the mammary gland of C/EBPβ^{null} mice is the sodium potassium chloride (NKCC1) cotransporter, which has been demonstrated previously to represent a marker of the ductal epithelium (17, 52, 53). Ductal morphogenesis is delayed in the mammary glands of NKCCl^{null} mice, and this effect is MEC autonomous (53). Both PrlR^{null} and Stat5ab-deficient mice fail to undergo lobuloalveolar development, perhaps because of the absence of lobuloalveolar progenitors (17, 54). The expression of NKCC1 is also retained in the ductal epithelium of these mice during pregnancy (53). Thus, it is conceivable that despite the presence of PR and PrlR, their ability to activate the appropriate cellular response is due to a deficiency in the lobuloalveolar progenitors in C/EBPβ^{null} mice.

Additional evidence for a change in cell fate in the mammary glands of C/EBPβ^{null} mice is the misexpression of SPRR2A, a marker of epidermal differentiation. SPRR2A is a protein normally expressed in the cornified layer of the epidermis and is involved in skin barrier function (30). No SPRR2A expression was detected by Northern blot analysis of mRNA from wild-type mice, or by immunohistochemical staining in the ductal epithelium, but high levels of expression were observed in the MECs of C/EBPβ^{null} mice in a nonuniform pattern. K6 expression was also observed in the MECs of C/EBPβ^{null} glands but not in the wild-type MECs or in transplanted tissue from PrlR^{null} or Stat5ab-deficient mice. The expression of K6 was more uniform than the expression of SPRR2A but was not detected in every cell. It has been proposed that K6/K14-positive MECs exist in mature mammary glands and may represent stem cells (32). Because K6 expression is normally observed in the body cells of terminal end buds (32, 33), this result suggests that deletion of C/EBPβ may prevent further differentiation of these ductal progenitors. It appears likely that the expression of these markers may represent a block in the normal cell fate determination and development pathway as a consequence of the germline deletion of C/EBPβ, which was not observed in the PrlR^{null} or Stat5ab-deficient mice.

Overall, these studies have illustrated the importance of appropriate receptor patterning in normal mammary gland development and have helped provide support for the model by which steroid hormones and Prl regulate lobuloalveolar development via a paracrine/juxtracrine mechanism. Disruptions in the pattern of these receptors and diminished proliferative responses were observed after the targeted deletion of several receptors, downstream signaling molecules and transcription factors, suggesting that this is a required mechanism for alveolar development. Although there are common defects between these knockout models, in particular increased PR and decreased proliferation, there are additional alterations in C/EBPβ^{null} mice that suggest an earlier role for this transcription factor in controlling MEC cell fate. The study of C/EBPβ^{null} mice continues to provide useful insights into the steps governing lobuloalveolar development. However, additional experiments are required to understand the precise molecular mechanisms mediating this complex developmental process.

MATERIALS AND METHODS

Animals and Tissue Collection

C/EBPβ^{null}, PR^{null}, PrlR^{null}, and Stat5ab-deficient mice have been described (2, 6, 9, 16). The genetic backgrounds of all the mice were as follows: C/EBPβ (C57BL/6 $\times$ 129Sv x

MF-1), PR (C57BL/6 × 129Sv), PrlR (129OlaHsd x 129SvPas), and Stat5ab (C57BL/6). All animal experimentation was conducted in accord with accepted standards of humane animal care.

The C/EBPβ mice were genotyped by PCR, rather than by Southern blot analysis as described previously (55). The primers for genotyping span the insertion site of the Neo cassette in the 3′ end of the C/EBPβ gene (55). This results in two different size products, either 367 bp for the wild-type allele or 1.3 kb for the mutant allele. Heterozygous animals will have both size bands. The forward primer was 5′-AGAAGACGGTGGACAAGCTG-3′ and the reverse primer was 5′-CTCGGTGCAGGTGCAGGT-3′. The 30-μl PCR contained 1.25 μM $MgCl_2$, 0.2 μM of each primer, and 1/20th vol of dimethylsulfoxide. Thirty cycles were performed using a denaturing step at 94 C for 30 sec, an annealing step at 55 C for 45 sec, and an extension time of 90 sec at 72 C.

Ovary-intact, nulliparous mice were treated for 48 h with a single interscapular sc injection of 17β-estradiol benzoate (1 μg) and P (1 mg) in 100 μl of sesame oil (all from Sigma, St. Louis, MO). For chronic E + P treatment, mice between 18 and 22 wk of age were treated for 21 d with E + P pellets as previously described (2). The transplantation of Stat5ab-deficient and $PrlR^{null}$ mammary tissue has been described elsewhere (17). Nine to 10 wk after transplantation into athymic NCr-nu/nu mice, an acute treatment of E + P was given. Two days later, both of the transplanted no. 4 inguinal mammary glands (Stat5ab-deficient and $PrlR^{null}$) and an endogenous no. 3 thoracic gland (control) were removed. Two hours before they were euthanized, all E + P-treated animals were injected ip with 0.3 mg BrdU per 10 g body weight (Amersham Pharmacia Biotech, Arlington Heights, IL). Tissues were fixed in 4% paraformaldehyde for 2 h at 4 C. Paraffin-embedded tissues were sectioned (5–7 μm) onto Probe-On Plus charged slides (Fisher Scientific, Pittsburgh, PA). Alternatively, mammary gland tissues were flash frozen in liquid nitrogen and stored at −80 C before cryostat sectioning. Ten-micron frozen sections were collected from all glands, mounted onto Superfrost Plus microscope slides (Fisher Scientific) and stored at −80 C.

RNA Analyses

Details for PrlR semiquantitative RT-PCR and ISH, including RNA preparation, primers and probes, have been described previously (24).

For Northern blot analyses, total RNA was first isolated from frozen mammary gland tissue using RNAzolB reagent (Tel-Test, Inc., Friendswood, TX). Poly(A) RNA isolation from total RNA was performed using the PolyATract I kit (Promega Corp., Madison, WI) according to manufacturer's instructions. Probes for PrlR-L, K18, SPRR2A, and IGFBP-5 were prepared by digesting the appropriate expression vectors, and the cyclophilin probe was purchased from Ambion, Inc. (Austin, TX). Inserts were labeled with α^{32}P-deoxy-ATP using the Prime-A-Gene kit (Promega Corp.). The blotting protocol was performed as previously described (4).

Radioactive ISH for IGF-II was performed on frozen sections as described previously (56). Nonradioactive ISHs were performed as for the radioactive ISH with the following modifications: the cRNA probe to IGFBP-5 was transcribed according to standard protocols (Roche Molecular Biochemicals, Indianapolis, IN) using a linearized mouse IGFBP-5 cDNA (57). Hybridizations were done overnight at 60 C with 400 ng/ml of digoxigenin-labeled IGFBP-5 cRNA probe. Sections were rinsed in Tris-buffered saline (TBS), blocked in 1× blocking reagent (Roche) in TBS and incubated in anti-DIG AP (1:500; Roche) in blocking reagent for 1 h at 37 C. Sections were then rinsed in TBS and incubated in detection buffer (100 mM Tris; 100 mM NaCl; 50 mM $MgCl_2$, pH 9.5) with levamisole (1.2 mg/ml) for 10 min. Sections were then incubated in nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt substrate system (Sigma) in the dark for 30 min. Slides were rinsed with water for 10 min, dehydrated through ethanols and xylenes, and coverslipped in Permount mounting media (Fisher Scientific).

RPAs were performed as described in the manufacturer's protocol (Ambion, Inc.). Twenty micrograms of total RNA were incubated with 3 fmol of ^{32}P-uridine triphosphate-labeled antisense IRS-1 (46) or β-Actin (Ambion, Inc.) riboprobes overnight at 55 C. Samples were run in triplicate and signal intensity was quantitated by PhosphorImager analysis using ImageQuant software (both from Amersham Biosciences, San Diego, CA).

Immunostaining

Sections were deparaffinized in xylenes, then rehydrated through a graded ethanol series. Indirect immunofluorescence for PR and BrdU and IRS-1 immunohistochemistry were performed as previously described (4, 46). Immunohistochemical staining was performed without antigen retrieval and used 5% goat serum (Sigma) in PBS as blocking buffer. Sections were incubated with the following primary antibodies overnight at room temperature: α-SPRR2 rabbit polyclonal antibody at 1:3000 (30), α-K6 (no. 66) rabbit polyclonal antibody at 1:5000 (kindly provided by Dennis Roop, Baylor College of Medicine, Houston, TX), and α-K14 rabbit polyclonal antibody at 1:10,000 (Covance, Richmond, CA). Immunoperoxidase staining was detected using the Vectastain Elite ABC kit and the diaminobenzidine substrate kit according to manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA).

Cell Counting and Statistical Analysis

Fluorescent images were digitally captured using an Olympus Corp. BX50 microscope connected to a Hamamatsu C5810 charge-coupled device. At least 6–16 individual 60× microscopic fields per sample were captured using the appropriate fluorescein isothiocyanate, Texas Red, and 4′,6 diamidino-2-phenylindole (DAPI) filters. The number of PR- and BrdU-positive MECs in a given field was expressed as a percentage of total number of DAPI-stained MECs. Statistical significance was determined by the Student's paired *t* test with all *P* values below 0.0001.

Immunoprecipitation and Western Blot Analyses

Frozen mammary gland tissues from three to four animals (ages 21–25 wk) for each genotype and treatment were pooled to prepare whole cell extracts as previously described (58). Immunoprecipitation assays were performed as previously described (58) using 1.5 mg of whole cell extract incubated with 800 ng of α-Stat5 rabbit polyclonal antibody (N-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antiphospho-tyrosine antibody PY20 (BD Biosciences, San Diego, CA) was used to evaluate the phosphorylation status of Stat5. The blot was then stripped and reprobed with the α-Stat5 rabbit polyclonal antibody used for the immunoprecipitation. IRS-1 protein was detected by Western blot analysis using 80 μg of whole cell extract per lane and the same antibody as used for immunohistochemistry, diluted 1:1000. MAPK p42/44 protein was used a loading control (1:1000; Cell Signaling Technology, Beverly, MA). Chemilumiescence was performed using SuperSignal reagents from Pierce Chemical Co. (Rockford, IL).

SSH PCR

The PCR-select cDNA Subtraction kit (CLONTECH Laboratories, Inc.) was used according to the manufacturer's instructions to screen for genes differentially regulated in the

mammary glands of mature, untreated C/EBPβ^{null} mice. Briefly, 2 μg of poly(A) RNA from either wild-type or C/EBPβ^{null} mammary glands (pooled tissue; 3–6 months old) were used to synthesize double-stranded cDNA. After subtraction and PCR amplification using nested primers, the cDNA inserts were cloned into pGEM-T Easy vectors (Promega Corp.), transformed into XL2-Blue ultracompetent cells (Stratagene, La Jolla, CA), and plated for blue/white color selection. Individual colonies (n = 672) were picked randomly and the inserts were PCR-amplified and screened by reverse Southern blot analysis using probes made from either enriched wild-type or C/EBPβ^{null} total cDNA. Approximately 60 clones showing detectable differences in expression levels by reverse Southern were then sequenced (59).

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